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(54) A recombinant delta 9 desaturase and a gene encoding the same.

(57) An isolated gene encoding Δ9 desaturase of cyanobacteria, an expression vector containing the same, a transformant transformed therewith and a recombinant Δ9 desaturase are provided, wherein said gene is useful for improving the composition of fatty acids of animals, plants and microorganisms by transformation and for producing animals, plants or microorganisms which tolerate low temperature.

EP 0 644 263 A2

Field of the Invention

The present invention relates to a recombinant Δ9 desaturase capable of converting stearic acid, a saturated fatty acid, that is linked to glycerolipid to oleic acid, an unsaturated fatty acid, and to an isolated gene encoding the same.

Background of the Invention

The Δ9 desaturase of cyanobacterium is an enzyme converting stearic acid linking to glycerolipid to oleic acid, and converting palmitic acid linking to C-1 of glycerol to palmitoleic acid.

In the cyanobacterium, the desaturation process of fatty acids has been shown to be initiated through the induction of the double bond into a carbon chain at Δ9 position, followed by Δ12 and then Δ6 or Δ15. The Δ9 desaturase is an important enzyme which is responsible for the first step of a series of desaturation reactions, and is associated with the reaction of introducing the double bond into a carbon chain of stearic acid or palmitic acid at Δ9, which are linked to glycerolipid. This reaction requires reducing power, which depends on ferredoxin and NADPH.

On the other hand, an enzyme introducing the double bond into stearic acid at Δ9, which is not linked to glycerolipid, has been reported as stearoyl CoA desaturase in cytoplasm of animals and as stearoyl ACP (acyl-carrier protein) desaturase in chloroplast of plants. The DNA sequence of these enzymes has been determined.

The Δ9 desaturase of cyanobacteria is characterized by converting palmitic acid or stearic acid linking to glycerolipid to unsaturated fatty acid, while above two Δ9 desaturases can not catalyze this reaction. To appreciate the determinating factors of its substrate specificity, Δ9 desaturase of several species of cyanobacterium should be analyzed at molecular level.

The phase transition temperature of biomembrane is dependant on the content of unsaturated fatty acids in polar lipid which consists of membrane; therefore, the phase transition temperature falls as the content of an unsaturated fatty acids increases. It has been reported that the amount of unsaturated fatty acids in cyanobacterium increases due to the lower temperature, suggesting that the composition of fatty acids in cell membrane is also associated with the low-temperature tolerance of plants. Thus, the expression of fatty acid desaturase is considered to be adjusted by low temperature. Approaches to the elucidation of the mechanisms of adjustment of expression demand isolating the associated gene(s).

For these reasons, the isolation of the gene of Δ9 desaturase of cyanobacteria has been required, however, there has been no report of the isolation of this gene with an exception of the isolation from Anabaena variabilis.

Detailed Description of the Invention

The present inventors have studied intensively for the purpose of analyzing the Δ9 desaturase of cyanobacteria at mdecular level and isolated genomic DNA clone of Δ9 desaturase of cyanobacteria Synechocystis sp.PCC6803, using genomic library of Synechocystis PCC6803, which led to the achievement of the present invention.

Therefore, the gist of the present invention lies in the Δ9 desaturase represented by the amino acid sequence shown in SEQ ID NO: 1 of Sequence Listing, and an isolated gene encoding the same.

The present invention will be discribed in more detail below.

In the present invention, cyanobacteria (e.g., Synechocystis ps.PCC6803) is grown photoautotrophically, cells are disrupted with glass beads, and the genomic DNA is extracted by phenol extraction and ethanol precipitation.

The entire genomic DNA is digested partially with restriction enzyme (e.g., Sau3A) and ligated to phage vector (e.g., λDASH II) to produce genomic library. The genomic library is screened by plaque hybridization, wherein the coding region of Δ9 desaturase (which may be abbreviated to desC(A) hereinafter) of cyanobacteria Anabaena variabilis is used as a probe. Phage DNA is extracted from positive plaque. After digestion with a restriction enzyme(s), Southan hybridization is performed using the 0.75 Kb.p. DNA fragment of desC(A) as a probe. The DNA fragments hybridized with probe DNA is sequenced by the dideoxy chain termination method.

The resultant base sequence of DNA fragments and amino acid sequence deduced therefrom are shown in SEQ ID NO: 1 of Sequence Listing.

The present invention also includes those derived from them through the deletion, replacement or addition of one or more amino acids or nucleotides from sequences shown in SEQ ID NO: 1 on condition

that the Δ9 unsaturase activity of a polypeptide coded by the DNA fragments is not affected.

The homology of the resultant gene with desC(A) is then examined to identify it as a new member of the Δ9 desaturase gene family. The activity of Δ9 desaturase can be measured after expression of the new gene in *E. coli*. The activity of Δ9 desaturase can be assayed by extracting the membrane of *E. coli* transformed with the isolated gene, adding ferredoxin, NADPH and stearic acid thereto and measuring the formation of oleic acid.

Knoell and Knappe, Eur. J. Biochem. 50, 245-252 (1974) reported that ferredoxin, an electron donor, was found in *E. coli*. Therefore, the activity can be confirmed by ligating the isolated gene to an expression vector for *E. coli*., transforming *E. coli*. with the vector inducing the expression of Δ9 desaturase-encoding DNA, and detecting the production of oleic acid.

The resultant gene of Δ9 desaturase, for example, when it is introduced into plant cells, can be ligated to a promoter which expresses in plant cells (e.g., CaMV 35S etc.) and a terminator (e.g., NOS etc.) to produce a chimeric gene, which is then ligated to *E. coli* plasmid (e.g., pUC19, pBR322, etc.), amplified, and introduced into a plant cell using an electroporation method. The gene can be also transferred into plant cells by means of Agrobacterium by ligating it to Ti plasmid or Ri plasmid of Agrobacterium or by using them as a binary vector. The transformation of the gene can lead to the change in composition of fatty acid and the improvement of tolerance to low temperature.

The gene encoding Δ9 desaturase of cyanobacteria of the present invention is useful for improving the composition of fatty acids of animals, plants and microorganisms and for producing animals, plants or organisms which tolerate low temperature by transformation.

The present invention is further illustrated by the following examples, while the invention is not limited by these examples as far as it falls within the scope of the gist.

EXAMPLE

(1) Extraction of Genomic DNA of Synechocystis PCC6803

A 300 ml of culture of Synechocystis PCC6803 (obtained from Pasteur Culture Collection) (the absorbance at 730 was between 5 and 10) was centrifuged at 4,500 x g for 6 minutes, and 1 - 2 g of cells were collected. To 1 g of cells, 2 ml of sodium iodide solution (4 g sodium iodide/2 ml distilled water) was added and suspended by shaking. The suspension was incubated at 37°C for 20 minutes and distilled water was added to a final volume of 40 ml, and the resulting solution was centrifuged at 10,000 x g for 10 minutes. The pellet was added to 10 ml of DNA-extraction-buffer (50 mM Tris-HCl (pH 8.5), 50 mM Sodium Chloride and 5 mM EDTA) and 1.5 ml of lysozyme solution (50 mg/ml), and was incubated at 37°C for 45 minutes. To the mixture was added 1 ml of 10% (w/v) N-lauroylsarcosine, and was incubated for another 20 minutes, while pipetting the disrupted cell solution several times in order to decrease the viscosity of the solution. To the disrupted cell solution was added 3 ml of ethidium bromide solution (10 mg/ml), and distilled water was added thereto to a final weight of 23 g. To the solution was added 21 g of cesium chloride and the mixture was centrifuged at 45,000 x g for 20 hours. After removing ethidium bromide from the solution containing recovered chromosome DNA by mixing with 1-buthanol repeatedly, the chromosome DNA solution was dialyzed against 4 litters of sterilized water for 90 minuets. After dialysis, the resulting DNA was extracted by an equal volume of phenol and then by an equal volume of chloroform, and was precipitated by ethanol. The precipitated DNA was collected by centrifugation and washed with 70% ethanol, dried, and dissolved in 100 μl of the buffer solution (10 mM Tris-HCl (pH 7.5)/0.1 mM EDTA).

(2) Screening of Genomic Library of Synechocystis PCC6803

The genomic DNA of Synechocystis PCC6803 was partially digested with a restriction endonuclease Sau3A, and was ligated into the BamHI site of phage vector-λDASH II. After infection of the phage containing genomic DNA of Synechocystis PCC6803 with *E. coli*, plaque hybridization was performed for 2,500 plaques using 0.75 kb DNA fragment of the coding region of desC gene of Anabaena variabilis as a probe. Twenty two clones were selected from the plaques which hybridized to the probe and the phage DNA was extracted. Entire genomic DNA of Synechocystis PCC6803 was digested with HindIII and analyzed by Southern hybridization using the same probe as described above, resulting in the detection of 6.0 kb band. Among the positive clones, the one which contained a 6.0Kb HindIII fragment was selected and the 6.0 kb HindIII fragment was subcloned into the HindIII site of plasmid Bluescript II KS(+).

(3) Isolation of Δ9 Desaturase Gene (desC) of Synechocystis PCC6803

The plasmid DNA containing the HindIII fragment was extracted for the preparation of a physical map using restriction endnucleases, PstI, BamHI, EcoRI, SpeI and ApaI. Moreover, the plasmid DNA was digested with the above restriction endnucleases and Southern hybridization was performed using the DNA fragment containing desC gene used in plaque hybridization as a probe for limiting a homologous region.

The limited region was sequenced by the dideoxy chain termination method to discover a protein coding region (abbribiated to "ORF" hereinafter) consisting of 975 bases. This gene showed 64% homology at an amino acid level with desC(A) of Anabaena variabilis. Comparisons of Δ12 desaturase gene among cyanobacterium, in which Synechocystis PCC6803 (Wada et al., Nature, 347, 200-203 (1990)) have 59% homolgy to Anabaena variabilis (Sakamoto et al., Plant. Mol. Biol. 24, 643-650 (1994)) and 57% homology to Synechococcus PCC7002 (Sakamoto et al., Plant. Mol. Biol. 24, 643-650 (1994)), reveal a high homology between the isolated ORF and desC(A) of Anabaena variabilis. The ORF shares 31% and 30% homology with stearoyl CoA desaturase of rat and yeast, respectively (rat: Thiede et al., J. Biol. Chem. 261, 13230-13235 (1986); yeast: Stukey et al., J. Biol. Chem., 265, 20144-20149). These results led to the conclusion that the isolated ORF is Δ9 desaturase gene of Synechocystis PCC6803 (desC). The base sequence of the Synechocystis PCC6803 desC and the amino acid sequence deduced therefrom are presented in SEQ ID NO: 1 of Sequence Listing.

(4) Construction of Expression Vector and Expression of Δ9 Desaturase in *E. coli*

The 0.5 kb fragment containing 5'-half region of des C obtained above was amplified by PCR and ligated into plasmid Bluescript II (pBSII). This DNA fragment was subcloned into the plasmid pBsII/H6 containing the 3'-half of the desC coding gegion and the resultant plasmid pBSII/desC was obtained. The pBSII/desC was digested with SpeI and a 1.1 kb DNA fragment containing a coding region was ligated into the NheI site of vector pET3a, which is located downstream from T7 bacteriophage promoter, and the pET3a/desC was obtained.

The pET3a/desC and, for comparison, pET3a which does not contain desC gene were transformed into *E. coli* BL21 (DE3) pLysS. Each transformant was cultured in LB medium containing stearic acid up to 0.6 of OD600 and further cultured for 1 hour with or without 1 mM IPTG. Cells were harvested by centrifugation, washed with 1.2% NaCl solution and collected again by centrifugation.

(5) Analysis of Fatty-Acid Composition of Individual Lipid Class of *E. coli*

Lipid was extracted from the collected *E. coli* by the method of Bligh and Dyer (Can. J. Biochem. Physiol., 37, 911-917 (1959)). The extracted lipid was separated into individual lipid class of PE (phosphatidyl ethanolamine), PG (phosphatidyl glycerol) and CL (cardiolipin) by silica gel thin-layer chromatography developed in $CHCl_3/CH_3OH/CH_3COOH$ (65:25:10). After separation, silica gel containing individual lipid class was scraped with a knife, and subjected to methanolysis in 5% HCl/methanol at 85 °C for 5.5 hours. The resultant methyl esters were extracted with 2 ml of n-hexane, concentrated and isolated by gas chromatography, and the content of individual lipid class was determined (Table 1).

The concentration of stearic acid in all lipid from *E. coli* grown in the medium without stearic acid was less than 1%, while in the medium with stearic acid the concentration was about 10%. As a control study, in *E. coli* transformed with pET3a, before and after the induction by IPTG, oleic acid did not increase and was less than 2% in any individual lipid class (Table 1). On the other hand, in *E. coli* transformed with pET3a/desC, the amount of oleic acid increased as a result of IPTG induction up to two or three times (6 - 10%) compared to that seen before induction (Table 1). The amount of palmitic acid, palmitoleic acid 16:1-(a) and vaccenic acid 18:1(11) did not change.

Table 1

Changes in fatty-acid composition of individual lipid classes by introduction of the *desC* genes into *E. coli*.

Lipid class	Fatty acid					
	14:0	16:0	16:1(9)	18:0	18:1(9)	18:1(11)
	(mol%)					
Before induction						
pET3a						
PE (78%)	2	31 ± 2	25 ± 1	14 ± 2	t	25 ± 1
PG (21%)	1	27 ± 2	17 ± 1	16 ± 1	1	36 ± 1
CL (1%)	1	32 ± 1	14 ± 1	19 ± 2	2	32 ± 2
pET3a/decC						
PE (80%)	3 ± 1	34 ± 1	24 ± 1	10 ± 1	2	26 ± 1
PG (19%)	1	31 ± 1	17 ± 1	10 ± 1	3 ± 1	36 ± 1
CL (1%)	0	30 ± 1	11 ± 1	10 ± 2	5 ± 1	39 ± 1
Induced by IPTG for 1 hr						
pET3a						
PE (82%)	4 ± 1	36 ± 3	24 ± 1	11 ± 2	t	23 ± 3
PG (17%)	1	30 ± 2	15 ± 1	14 ± 1	1	39 ± 1
CL (1%)	1	36 ± 1	12 ± 1	16 ± 2	1	33 ± 2
pET3a/desC						
PE (74%)	3 ± 1	33 ± 1	24 ± 1	9 ± 1	6 ± 1	24 ± 1
PG (21%)	1	30 ± 1	19 ± 1	8 ± 1	10 ± 1	31 ± 1
CL (5%)	1	27 ± 1	18 ± 1	8 ± 1	10 ± 1	36 ± 1

Values were obtained from three independent cultures.
t: Trace (less than 0.5%).

(6) Analysis of Fatty Acid Composition at Each Bind Site of Glycerol Skelton

By the method as described above, fatty acids were extracted from *E. coli* induced by IPTG, and PE and PG were separated by silica gel thin-layer chromatography. These were selectively hydrolysed by the method of Fischer et al. (Hoppe-Seyler's Z. Physiol. Chem. 354, 1151-1123(1973)) using lipase from Rhizopus delemar. After methanolysis, the amount of fatty acid methylester(s) was determined by gas chromatography.

In control experiment where *E. coli* was transformed with pET3a, the rate of oleic acid in fatty acids linking to C-1 position of glycerol skelton was less than 0.5% in either cases of PE and PG. On the other hand, in *E. coli* transformed with pET3a/desC, the rate of oleic acid in fatty acids linking to C-1 position of glycerol skelton increased to 11% and 18% in the cases of PE and PG, respectively (Table 2). However, there is no difference in C-2 position. The rate of palmitic acid, palmitoleic acid and vaccenic acid did not change.

These results indicate that the isolated gene encodes Δ9 desaturase which converts stearic acid linking to C-1 position of phospholipids to an unsaturated acid, regardless of polar residue.

Table 2

Positional distribution of fatty acids in individual lipid classes of *E. coli* cells transformed with the *desC* genes						
Lipid class (position)	Fatty acid					
	14:0	16:0	16:1(9)	18:0	18:1(9)	18:1(11)
	(mol%)					
pET3a						
PE (C-1)	1	68	6	16	t	5
(C-2)	3	4	42	6	1	41
PG (C-1)	1	51	12	16	t	20
(C-2)	1	8	18	11	3	58
pET3a/desC						
PE (C-1)	1	61	7	9	11	11
(C-2)	3	5	41	9	1	37
PG (C-1)	1	51	16	1	18	14
(C-2)	1	7	22	18	2	48
Values were obtained from three independent cultures. The deviation of the values was within 2%. t: Trace (less than 0.5%).						

REFERENCE EXAMPLE: Isolation of Gene Encoding Anabaena Variabilis Δ9 Desaturase

(1) Extraction of Genomic DNA

A 300 ml of the culture of Anabaena variabilis strain M-3 (obtained from Institute of Applied Microbiology University of Tokyo) (absorbance at 730 was between 5 and 10) was centrifuged at 4,500 x g for 6 minutes, and 1 - 2 g of cells were collected. To 1 g of cells, 2 ml of sodium iodide solution (4 g sodium iodide/2 ml distilled water) was added and suspended by shaking. The suspension was incubated at 37°C for 20 minutes and distilled water was added to a final volume of 40 ml, and the resulting solution was centrifuged at 10,000 x g for 10 minutes. The pellet was resuspended in 10 ml of DNA-extraction-buffer (50 mM Tris-HCl (pH 8.5), 50 mM Sodium Chloride and 5 mM EDTA) and 5 ml of lysozyme solution (50 mg/ml), and was incubated at 37°C for 45 minutes. To the mixture was added 1 ml of 10% (w/v) N-lauroylsarcosine, and was incubated again for another 20 minutes, while pipetting the disrupted cell solution several times in order to decrease the viscosity of the solution. To the disrupted cell solution was added 3 ml of ethidium bromide solution (10 mg/ml), and distilled water was added thereto to a final weight of 23 g. To the solution was added 21 g of cesium chloride and the mixture was centrifuged at 45,000 x g for 20 hours. After removing ethidium bromide from the solution containing recovered chromosome DNA by mixing with 1-buthanol repeatedly, the chromosome DNA solution was dialyzed against 4 litters of sterilized water for 90 minutes. After dialysis, the resulting DNA was extracted by an equal volume of phenol and then by an equal volume of chloroform, and was precipitated by ethanol. The precipitated DNA was collected by centrifugation and washed by 70% ethanol, dried, and dissolved in 100 µl of the buffer (10 mM Tris-HCl (pH 7.5)/0.1 mM EDTA).

(2) The Isolation of Δ12 Desaturase gene (desA) of Anabaena Variabilis

Anabaena Variabilis DNA obtained as described above was partially digested with a restriction endonuclease Sau3A, and was ligated into the BamHI site of phage vector-λDASH II. After infection of the λphage including gemonic DNA of Anabaena variabilis with *E. coli*, plaque hybridization was performed for 3.5 x 10^3 plaques using 1.1 kb HincII-SpeI DNA fragment containing Δ12 desaturase gene (desA) of Synechocystis PCC6803 as a probe. Three clones were selected randomly from the plaques which hybridized to the probe. The phage DNA was extracted, digested with restriction endonuclease HincII and

analyzed by Southern hybridization using the same probe as described above. In both phage DNA, 2.1 kb of bands hybridizing to the probe were found and one of them was examined for further identification of the gene.

The identification of the gene was performed as follows: Phage DNA was digested with restriction endonuclease EcoRI and Southern hybridization was performed to prove that a 7 kb fragment was homologous to the probe. This 7 kb fragment was ligated into the EcoRI site of shuttle vector pUC303 (Kuhlemier et al., Plasmid 10, 156-163 (1983)) between *E. coli* and Synechococcus PCC7942 to obtain pUC303/7-kb.

Since Synechococcus PCC7942 has fatty acids of 16:0, 16:1, 18:0 and 18:1, but dose not have 16:2 and 18:2, this strain is considered to lack in Δ12 desaturase gene. It has been reported that introduction of desA gene of Synechococcus PCC6803 to Synechococcus PCC7942 led to production of unsaturated fatty acid of 16:2 and 18:2 (Wada et al., 1990 ibid). Synechococcus PCC7942 was then transformed with pUC303/7-kb by the method of Williams & Szalay, Gene, 24, 37-51 (1983). PCC7942 was cultured in 50 ml of BG-11 liquid medium up to 5-8 x 10^7/ml and centrifuged at 4,500 x g for 10 minutes at room temperature. The precipitated cells were washed again with BG-11 medium, collected by centrifugation and suspended in BG-11 medium to a final concentration of 1-2 x 10^9 cells/ml. To 0.1 ml of the cell suspension was added 0.1 μg of DNA and shaked gently in the light for 1 hour. The transformed cells were grown in the BG-11 agar medium containing 10 μg/ml of streptomycin, at the density of 1-5 x 10^7 cells/plate in the dark at 30 • C for 16 hours and further grown in the light for 8 hours. After 0.5 ml of 1 mg/ml of streptomycin was added dropwise to the agar medium, streptomycin resistant transformant cells producing green signal were chosen.

The transformant was grown in 100 ml of BG-11 medium, centrifuged at 4,500 x g for 10 minutes and lyophilized. The dried cells were added to 10 ml of methanol containing 5% HCl (w/w) and heated at 85 • C for 2.5 hours for methanolysis. The resulting fatty acid methylester was extracted with 3 ml of n-hexane, three times. After removal of hexane by evaporation, the sample was dissolved again in 0.1 ml of hexane. An aliquot of the sample solution was taken and used for analysis of fatty acid methylester composition by gas chromatography.

Synechococcus PCC7942 wild strain dose not have unsaturated fatty acid of 18:2, while the cell transformed with pUC303/7-kb produced 1% of 18:2 unsaturated fatty acid in total fatty acid, therefore it was concluded that desA gene of Anabaena variabilis was present in 7-kb EcoRI fragment.

Physical map was designed by digesting 7-kb EcoRI fragment with restriction endonuclease ClaI, SpeI and HindIII. Moreover, a region homologous to desA of Synechocystis PCC6803 was identified by Southern hybridization and sequenced by the dideoxy chain termination method. Since an Open reading frame (ORF) composed of 1053 bases was found and three regions highly homologous to desA of Synechocystis PCC6803 (more than 80%) were noted in the amino acid sequence of ORF, it was concluded that this ORF was desA gene of Anabaena variabilis.

(3) Isolation of Δ9 Desaturase Gene (desC(A)) of Anabaena Variabilis

Determination of base sequence of 5' upstream Anabaena variabilis desA gene revealed an open reading frame (ORF) which was composed of 819 bases within about 1.2 kb. Since the amino acid sequence of this ORF product had 31% and 29% homology with stearoyl CoA desaturase of rat and yeast respectively, it was concluded that the ORF was Δ9 desaturase gene (desC(A)) of Anabaena variabilis. The base sequence of Anabaena variabilis desC(A) and the amino acid sequence deduced therefrom are presented in SEQ ID NO: 2 of Sequence Listing. SEQ ID NO: 3 corresponds to the amino acid sequence of SEQ ID NO: 1, and SEQ ID NO: 4 corresponds to the amino acid sequence of SEQ ID NO: 2.

SEQUENCE LISTING

(1) GENERAL INFORMATION:

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(ii) TITLE OF INVENTION: A DELTA-9 DESATURASE AND A GENE ENCODING THE SAME

(iii) NUMBER OF SEQUENCES: 4

(iv) COMPUTER READABLE FORM:
(A) MEDIUM TYPE: Floppy disk
(B) COMPUTER: IBM PC compatible
(C) OPERATING SYSTEM: PC-DOS/MS-DOS
(D) SOFTWARE: PatentIn Release #1.0, Version #1.25 (EPO)

(vi) PRIOR APPLICATION DATA:
(A) APPLICATION NUMBER: JP 236720/1993
(B) FILING DATE: 22-SEP-1993

(2) INFORMATION FOR SEQ ID NO: 1:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 957 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: double
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(vi) ORIGINAL SOURCE:
(A) ORGANISM: Synechocystis PCC6803

(ix) FEATURE:
(A) NAME/KEY: CDS
(B) LOCATION: 1..954

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 1:

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ATG TTA AAC CCA TTA AAC ATT GAA TAC CTA TAT TTA AGC AAA CTT TTT        48
Met Leu Asn Pro Leu Asn Ile Glu Tyr Leu Tyr Leu Ser Lys Leu Phe
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          1               5                  10                  15

GAC AAT AGT TTA ATC GTT TTT AAC AAG CGC CAA TTA TTC CGT TTT TTC          96
Asp Asn Ser Leu Ile Val Phe Asn Lys Arg Gln Leu Phe Arg Phe Phe
                20                  25                  30

GTT AGG TTT TTT TTC ATG ACT GCT GCT CTT CCC AAC GAT TCC AAG CCC         144
Val Arg Phe Phe Phe Met Thr Ala Ala Leu Pro Asn Asp Ser Lys Pro
            35                  40                  45

AAG TTG ACT CCA GCT TGG ACT GTG ATC TTC TTT TTT ACC TCC ATT CAT         192
Lys Leu Thr Pro Ala Trp Thr Val Ile Phe Phe Phe Thr Ser Ile His
        50                  55                  60

TTG GTG GCC CTG TTG GCT TTC CTG CCC CAG TTT TTC AGT TGG AAA GCA         240
Leu Val Ala Leu Leu Ala Phe Leu Pro Gln Phe Phe Ser Trp Lys Ala
    65                  70                  75                  80

GTG GGG ATG GCT TTC TTG CTC TAT GTA ATT ACC GGC GGC ATT GGC ATT         288
Val Gly Met Ala Phe Leu Leu Tyr Val Ile Thr Gly Gly Ile Gly Ile
                85                  90                  95

ACT TTA GGT TTT CAC CGT TGT ATT TCC CAC CGC AGT TTC AAT GTT CCT         336
Thr Leu Gly Phe His Arg Cys Ile Ser His Arg Ser Phe Asn Val Pro
            100                 105                 110

AAA TGG TTA GAG TAT ATT TTC GTA ATC TGT GGC ACC CTA GCC TGT CAG         384
Lys Trp Leu Glu Tyr Ile Phe Val Ile Cys Gly Thr Leu Ala Cys Gln
        115                 120                 125

GGG GGC GTA TTT GAG TGG GTC GGC TTA CAC CGT ATG CAC CAC AAA TTT         432
Gly Gly Val Phe Glu Trp Val Gly Leu His Arg Met His His Lys Phe
    130                 135                 140

TCT GAC ACC ACC CCG GAT CCC CAC GAT TCT AAT AAG GGT TTT TGG TGG         480
Ser Asp Thr Thr Pro Asp Pro His Asp Ser Asn Lys Gly Phe Trp Trp
145                 150                 155                 160

AGT CAC ATC GGC TGG ATG ATG TTT GAA ATT CCT GCT AAA GCT GAT ATT         528
Ser His Ile Gly Trp Met Met Phe Glu Ile Pro Ala Lys Ala Asp Ile
                165                 170                 175

CCC CGC TAC ACC AAG GAT ATC CAA GAC GAT AAA TTT TAT CAA TTT TGC         576
Pro Arg Tyr Thr Lys Asp Ile Gln Asp Asp Lys Phe Tyr Gln Phe Cys
            180                 185                 190

CAG AAT AAT CTA ATT CTT ATC CAG GTC GCC CTA GGC TTG ATT TTA TTT         624
Gln Asn Asn Leu Ile Leu Ile Gln Val Ala Leu Gly Leu Ile Leu Phe
        195                 200                 205

GCC TTA GGG GGC TGG CCC TTC GTT ATT TGG GGC ATT TTT GTC CGC CTA         672
Ala Leu Gly Gly Trp Pro Phe Val Ile Trp Gly Ile Phe Val Arg Leu
    210                 215                 220

GTG TTT GTT TTC CAC TTC ACT TGG TTT GTC AAC AGT GCC ACC CAT AAG         720
Val Phe Val Phe His Phe Thr Trp Phe Val Asn Ser Ala Thr His Lys
225                 230                 235                 240

TTC GGC TAC GTT AGC CAT GAA TCC AAT GAT TAT TCC CGC AAT TGT TGG         768
Phe Gly Tyr Val Ser His Glu Ser Asn Asp Tyr Ser Arg Asn Cys Trp
                245                 250                 255

TGG GTA GCA TTG TTA ACT TTC GGT GAA GGT TGG CAC AAT AAT CAC CAC         816
```

```
Trp Val Ala Leu Leu Thr Phe Gly Glu Gly Trp His Asn Asn His His
          260                 265                 270

GCC TAT CAG TAC TCT GCT CGC CAT GGT TTG CAA TGG TGG GAA GTG GAT      864
Ala Tyr Gln Tyr Ser Ala Arg His Gly Leu Gln Trp Trp Glu Val Asp
      275                 280                 285

TTA ACT TGG ATG ACC ATT AAA TTC CTA TCT TTG CTG GGG TTA GCC AAG      912
Leu Thr Trp Met Thr Ile Lys Phe Leu Ser Leu Leu Gly Leu Ala Lys
  290                 295                 300

GAT ATT AAA CTT CCT CCG GAA ACT GCG ATG GCC AAC AAA GCC              954
Asp Ile Lys Leu Pro Pro Glu Thr Ala Met Ala Asn Lys Ala
305                 310                 315

TAG                                                                 957
```

(2) INFORMATION FOR SEQ ID NO: 2:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 819 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: double
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(vi) ORIGINAL SOURCE:
(A) ORGANISM: Anabaena variabilis

(ix) FEATURE:
(A) NAME/KEY: CDS
(B) LOCATION: 1..816

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 2:

```
ATG ACA ATT GCT ACT TCA ACT AAA CCT CAA ATC AAC TGG GTA AAT ACC       48
Met Thr Ile Ala Thr Ser Thr Lys Pro Gln Ile Asn Trp Val Asn Thr
  1               5                  10                  15

CTA TTT TTC CTT GGG CTA CAC ATC GGC GCT TTG TTT GCC TTT ATC CCT       96
Leu Phe Phe Leu Gly Leu His Ile Gly Ala Leu Phe Ala Phe Ile Pro
            20                  25                  30

AGT AAC TTC AGC TGG GCG GCA GTT GGT GTG GCT TTA TTG CTT TAC TGG      144
Ser Asn Phe Ser Trp Ala Ala Val Gly Val Ala Leu Leu Leu Tyr Trp
        35                  40                  45

ATC ACT GGT GGT TTG GGT ATT ACC TTA GGC TTT CAT CGC CTT GTT ACC      192
Ile Thr Gly Gly Leu Gly Ile Thr Leu Gly Phe His Arg Leu Val Thr
    50                  55                  60

CAC CGC AGT TTT CAG ACT CCC AAG TGG TTG GAA TAT TTT CTA GTG CTT      240
His Arg Ser Phe Gln Thr Pro Lys Trp Leu Glu Tyr Phe Leu Val Leu
65                  70                  75                  80

TGC GGG ACT CTC GCT TGT CAA GGA GGG CCA ATC GAG TGG GTC GGT ACA      288
Cys Gly Thr Leu Ala Cys Gln Gly Gly Pro Ile Glu Trp Val Gly Thr
                85                  90                  95
```

```
CAT CGC ATT CAT CAT TTA CAT TCC GAT ACT GAT CCA GAT CCC CAT GAT      336
His Arg Ile His His Leu His Ser Asp Thr Asp Pro Asp Pro His Asp
            100                 105                 110

TCT AAT AAA GGT TTC TGG TGG AGC CAT ATT GGT TGG CTA ATT TAT CAC      384
Ser Asn Lys Gly Phe Trp Trp Ser His Ile Gly Trp Leu Ile Tyr His
        115                 120                 125

TCT CCC TCC CAC GCT GAT GTT CCT CGG TTC ACC AAA GAT ATT GCC GAA      432
Ser Pro Ser His Ala Asp Val Pro Arg Phe Thr Lys Asp Ile Ala Glu
    130                 135                 140

GAC CCA GTC TAT CAG TTT TTA CAG AAA TAT TTC ATT TTT ATC CAG ATT      480
Asp Pro Val Tyr Gln Phe Leu Gln Lys Tyr Phe Ile Phe Ile Gln Ile
145                 150                 155                 160

GCT CTG GGG TTG TTG CTG TTA TAT CTA GGC GGG TGG TCT TTT GTG GTC      528
Ala Leu Gly Leu Leu Leu Leu Tyr Leu Gly Gly Trp Ser Phe Val Val
                165                 170                 175

TGG GGA GTT TTC TTT CGC ATC GTT TGG GTT TAC CAC TGT ACT TGG TTG      576
Trp Gly Val Phe Phe Arg Ile Val Trp Val Tyr His Cys Thr Trp Leu
            180                 185                 190

GTA AAC AGC GCT ACC CAT AAG TTT GGC TAC CGC ACC TAT GAT GCT GGT      624
Val Asn Ser Ala Thr His Lys Phe Gly Tyr Arg Thr Tyr Asp Ala Gly
        195                 200                 205

GAC AGA TCC ACT AAC TGT TGG TGG GTA GCT GTC CTA GTG TTT GGT GAA      672
Asp Arg Ser Thr Asn Cys Trp Trp Val Ala Val Leu Val Phe Gly Glu
    210                 215                 220

GGT TGG CAC AAC AAC CAC CAC GCT TTT CAA TAT TCA GCT CGT CAC GGG      720
Gly Trp His Asn Asn His His Ala Phe Gln Tyr Ser Ala Arg His Gly
225                 230                 235                 240

TTG GAA TGG TGG GAA GTT GAT CTG ACT TGG ATG ACA GTG CAA TTG CTG      768
Leu Glu Trp Trp Glu Val Asp Leu Thr Trp Met Thr Val Gln Leu Leu
                245                 250                 255

CAA ATA CTC GGT TTA GCA ACT AAT GTC AAA CTA GCA GAC AAA AAG CAG      816
Gln Ile Leu Gly Leu Ala Thr Asn Val Lys Leu Ala Asp Lys Lys Gln
            260                 265                 270

TAA                                                                 819
```

(2) INFORMATION FOR SEQ ID NO: 3:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 318 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 3:

```
Met Leu Asn Pro Leu Asn Ile Glu Tyr Leu Tyr Leu Ser Lys Leu Phe
 1               5                  10                  15

Asp Asn Ser Leu Ile Val Phe Asn Lys Arg Gln Leu Phe Arg Phe Phe
            20                  25                  30
```

```
Val Arg Phe Phe Phe Met Thr Ala Ala Leu Pro Asn Asp Ser Lys Pro
        35                  40                  45

Lys Leu Thr Pro Ala Trp Thr Val Ile Phe Phe Phe Thr Ser Ile His
    50                  55                  60

Leu Val Ala Leu Leu Ala Phe Leu Pro Gln Phe Phe Ser Trp Lys Ala
65                  70                  75                  80

Val Gly Met Ala Phe Leu Leu Tyr Val Ile Thr Gly Gly Ile Gly Ile
                85                  90                  95

Thr Leu Gly Phe His Arg Cys Ile Ser His Arg Ser Phe Asn Val Pro
            100                 105                 110

Lys Trp Leu Glu Tyr Ile Phe Val Ile Cys Gly Thr Leu Ala Cys Gln
        115                 120                 125

Gly Gly Val Phe Glu Trp Val Gly Leu His Arg Met His His Lys Phe
    130                 135                 140

Ser Asp Thr Thr Pro Asp Pro His Asp Ser Asn Lys Gly Phe Trp Trp
145                 150                 155                 160

Ser His Ile Gly Trp Met Met Phe Glu Ile Pro Ala Lys Ala Asp Ile
                165                 170                 175

Pro Arg Tyr Thr Lys Asp Ile Gln Asp Asp Lys Phe Tyr Gln Phe Cys
            180                 185                 190

Gln Asn Asn Leu Ile Leu Ile Gln Val Ala Leu Gly Leu Ile Leu Phe
        195                 200                 205

Ala Leu Gly Gly Trp Pro Phe Val Ile Trp Gly Ile Phe Val Arg Leu
    210                 215                 220

Val Phe Val Phe His Phe Thr Trp Phe Val Asn Ser Ala Thr His Lys
225                 230                 235                 240

Phe Gly Tyr Val Ser His Glu Ser Asn Asp Tyr Ser Arg Asn Cys Trp
                245                 250                 255

Trp Val Ala Leu Leu Thr Phe Gly Glu Gly Trp His Asn Asn His His
            260                 265                 270

Ala Tyr Gln Tyr Ser Ala Arg His Gly Leu Gln Trp Trp Glu Val Asp
        275                 280                 285

Leu Thr Trp Met Thr Ile Lys Phe Leu Ser Leu Leu Gly Leu Ala Lys
    290                 295                 300

Asp Ile Lys Leu Pro Pro Glu Thr Ala Met Ala Asn Lys Ala
305                 310                 315
```

(2) INFORMATION FOR SEQ ID NO: 4:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 272 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 4:

```
Met Thr Ile Ala Thr Ser Thr Lys Pro Gln Ile Asn Trp Val Asn Thr
 1               5                  10                  15

Leu Phe Phe Leu Gly Leu His Ile Gly Ala Leu Phe Ala Phe Ile Pro
            20                  25                  30

Ser Asn Phe Ser Trp Ala Ala Val Gly Val Ala Leu Leu Leu Tyr Trp
        35                  40                  45

Ile Thr Gly Gly Leu Gly Ile Thr Leu Gly Phe His Arg Leu Val Thr
    50                  55                  60

His Arg Ser Phe Gln Thr Pro Lys Trp Leu Glu Tyr Phe Leu Val Leu
65                  70                  75                  80

Cys Gly Thr Leu Ala Cys Gln Gly Gly Pro Ile Glu Trp Val Gly Thr
                85                  90                  95

His Arg Ile His His Leu His Ser Asp Thr Asp Pro Asp Pro His Asp
            100                 105                 110

Ser Asn Lys Gly Phe Trp Trp Ser His Ile Gly Trp Leu Ile Tyr His
        115                 120                 125

Ser Pro Ser His Ala Asp Val Pro Arg Phe Thr Lys Asp Ile Ala Glu
    130                 135                 140

Asp Pro Val Tyr Gln Phe Leu Gln Lys Tyr Phe Ile Phe Ile Gln Ile
145                 150                 155                 160

Ala Leu Gly Leu Leu Leu Leu Tyr Leu Gly Gly Trp Ser Phe Val Val
                165                 170                 175

Trp Gly Val Phe Phe Arg Ile Val Trp Val Tyr His Cys Thr Trp Leu
            180                 185                 190

Val Asn Ser Ala Thr His Lys Phe Gly Tyr Arg Thr Tyr Asp Ala Gly
        195                 200                 205

Asp Arg Ser Thr Asn Cys Trp Trp Val Ala Val Leu Val Phe Gly Glu
    210                 215                 220

Gly Trp His Asn Asn His His Ala Phe Gln Tyr Ser Ala Arg His Gly
225                 230                 235                 240

Leu Glu Trp Trp Glu Val Asp Leu Thr Trp Met Thr Val Gln Leu Leu
                245                 250                 255

Gln Ile Leu Gly Leu Ala Thr Asn Val Lys Leu Ala Asp Lys Lys Gln
            260                 265                 270
```

Claims

1. A recombinant Δ9 desaturase which is represented by the amino acid sequence shown in SEQ ID NO: 1 of Sequence Listing.

2. An isolated gene encoding the Δ9 desaturase as claimed in Claim 1.

3. The gene as claimed in Claim 2, which is represented by the base sequence shown in SEQ ID NO: 1 of Sequence Listing.

4. A recombinant vector capable of expressing a polypeptide coded by the gene as claimed in Claim 2 or 3.

5. A transformant obtained by transforming a host cell with the recombinant vector as claimed in Claim 4.

6. A method for producting the recombinant Δ9 desaturase as claimed in Claim 1, which comprises growing the transformant as claimed in Claim 5 in a medium and recovering the expression product.

(19) Europäisches Patentamt
European Patent Office
Office européen des brevets

(11) EP 0 644 263 A3

(12) EUROPEAN PATENT APPLICATION

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(54) A recombinant delta 9 desaturase and a gene encoding the same

(57) An isolated gene encoding Δ9 desaturase of cyanobacteria, an expression vector containing the same, a transformant transformed therewith and a recombinant Δ9 desaturase are provided, wherein said gene is useful for improving the composition of fatty acids of animals, plants and microorganisms by transformation and for producing animals, plants or microorganisms which tolerate low temperature.

EP 0 644 263 A3

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European Patent Office

EUROPEAN SEARCH REPORT

Application Number
EP 94 11 4957

DOCUMENTS CONSIDERED TO BE RELEVANT

Category	Citation of document with indication, where appropriate, of relevant passages	Relevant to claim	CLASSIFICATION OF THE APPLICATION (Int.Cl.6)
A	PLANT PHYSIOLOGY, vol. 92, 1990, pages 1062-1069, XP002015048 WADA H AND MURATA N.: "Temperature-Induced Changes in the Fatty Acid Composition of Cyanobacterium Synechocystis PCC6803" The whole document	1-6	C12N15/53 C12N9/02 C12N15/70 C12N1/21 C12N15/82
A	EP-A-0 550 162 (PIONEER HI BRED INT) 7 July 1993 claim 1 and 2 * page 2, line 1-4 - page 3, line 15-20 *	1-6	
A	WO-A-91 13972 (CALGENE INC) 19 September 1991 Abstract and Claims 1, 2, 16 and 27 * page 6, line 4-11 - page 9, line 4-17 *	1-6	
A,D	NATURE, vol. 347, 13 September 1990, LONDON, pages 200-203, XP002014843 WADA H., GOMBOS Z. MURATA N.: "Enhancement of chilling tolerance of a cyanobacterium by genetic manipulation of fatty acid desaturation" The Whole Document	1-6	TECHNICAL FIELDS SEARCHED (Int.Cl.6) C12N
T	JOURNAL OF BIOLOGICAL CHEMISTRY, vol. 269, no. 41, 14 October 1994, pages 25576-25580, XP002014844 SAKAMOTO T. ET AL.: "Delta 9 Acyl-Lipid Desaturases of Cyanobacteria" The Whole Document	1-6	

The present search report has been drawn up for all claims

Place of search	Date of completion of the search	Examiner
BERLIN	3 October 1996	Panzica, G

CATEGORY OF CITED DOCUMENTS

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